

# Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces

(combinatorial immunoglobulin repertoire/filamentous bacteriophage/surface expression/coat protein VIII/catalytic antibodies)

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**ABSTRACT** We describe a method based on a phagemid vector with helper phage rescue for the construction and rapid analysis of combinatorial antibody Fab libraries. This approach should allow the generation and selection of many monoclonal antibodies. Antibody genes are expressed in concert with phage morphogenesis, thereby allowing incorporation of functional Fab molecules along the surface of filamentous phage. The power of the method depends upon the linkage of recognition and replication functions and is not limited to antibody molecules.

Various molecular genetic approaches have been devised to capture the vast immunological repertoire and to generate and overexpress its antibodies (1–10). This capability is particularly pertinent for locating catalytic antibodies, where often many different binding modes need to be sampled in order to find efficient catalysts (reviewed in ref. 11). For some catalytic antibodies the precise positioning of nucleophiles or acid/base residues has proved to be critical (11). Our approach has been to generate combinatorial libraries in which large numbers of heavy and light chains are randomly combined to generate functional antibodies. The combinatorial method is useful not only because of the large number of antibodies sampled but also because it can be used to bring cofactors such as metals into the antibody binding pocket via the heavy or light chain or both. This method has been shown to be successful for the generation of diverse high-affinity antibodies to haptens (10), virus particles (12), and protein antigens (13, 14). From immunized animals it has been possible to recapitulate functional molecules that appear during the natural immune response as well as to harvest catalytic antibodies.

The biggest challenge for the methodology is the development of powerful methods to access very large combinatorial libraries. One of the most interesting approaches would be to link the recognition element and the instructions for its production. Recently, this has been accomplished for peptide libraries, proteins (15–19), and a single-chain antibody (20). Here we describe an approach using a combinatorial vector for the expression of functional antibody Fab molecules along the entire length of filamentous phage particles. The method depends upon assembly of the antibody molecules in the periplasm in concert with phage morphogenesis.

## METHODS

**Vector Construction and Phage Preparation.** The *lacZ* promoter, ribosome binding site, *pelB* leader sequences, sites for directional cloning of the PCR-amplified antibody fragments,

and chloramphenicol- or ampicillin-resistance markers were used to modify pBluescript phagemid vector (Stratagene). Clone 2b, encoding Fab isolated from the original *p*-nitrophenyl phosphonamidate (NPN) combinatorial library (10) was modified to remove the region encoding the decapeptide tag by digestion with restriction enzymes *EcoRI* and *Spe I*, and in its place the M13 phage coat protein VIII (cpVIII) fusion-protein gene was inserted. The Fab-encoding fragment (*Xho I*–*Xba I*) was inserted into the vector to produce pCBAK8. Transformed *Escherichia coli* XL1-Blue cells were selected on LB plates (chloramphenicol, 30 µg/ml) and grown in liquid culture at 37°C in super broth (enriched with 4-morpholinesulfonic acid) with antibiotic selection for the phagemid and the F' episome (chloramphenicol, 30 µg/ml, and tetracycline, 50 µg/ml, respectively). Cells were diluted to an OD<sub>600</sub> of 0.4 in super broth and incubated with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 1 hr. Helper phage R408 or VCS M13 (phage/cell ratio, 10–20:1) was added, and the cultures were grown for a further 2 hr at 37°C. The cells were pelleted, and supernatants containing phage were assayed directly unless otherwise stated.

**Electron Microscopy.** To localize functional Fab molecules the binding to antigen labeled with colloidal gold was studied. Phage-containing supernatants and bacterial cells were spotted onto Formvar-coated grids. In some experiments grids were coated with cells and infected with phage *in situ*. Subsequently grids were blocked with 1% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at pH 7.2, washed, and incubated with 5- to 7-nm colloidal gold particles coated with BSA–NPN hapten conjugate. The grids were washed to remove excess gold particles, negatively stained with uranyl acetate, and visualized by electron microscopy.

**Phage ELISA.** Microtitration plates were coated with NPN–BSA conjugate (0.1 ml, 1 µg/ml in 0.1 M Tris, pH 9.2) and blocked with 1% BSA in PBS. Serial 2-fold dilutions of phage (0.1 ml) were added to the precoated microtitration plate and incubated for 3 hr at ambient temperature or 16 hr at 4°C. The plates were washed with PBS and goat anti-κ alkaline phosphatase conjugate was added (0.1 ml diluted 1:1000 in PBS containing 0.1% BSA) and incubated for 2 hr at ambient temperature. The plates were washed in PBS and substrate was added (0.1 ml, 0.1% *p*-nitrophenyl phosphate in 0.1 M Tris, pH 9.5, containing 50 mM MgCl<sub>2</sub>). After incubation at 37°C for signal development, OD<sub>400</sub> was determined. Competition assay were performed with the addition of increasing amounts of free NPN hapten.

**Antigen-Specific Precipitation of Phage.** Phage-containing supernatant (1 ml) was incubated with BSA–NPN conjugate

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Abbreviations: cpVIII and cpIII, coat proteins VIII and III derived from M13 phage; NPN, *p*-nitrophenyl phosphonamidate; BSA, bovine serum albumin.

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(10  $\mu$ l, 2 mg/ml) for 18 hr at 4°C. The mixture was then pelleted by centrifugation at 3000 rpm on a benchtop centrifuge and the appearance of precipitate was noted. Helper phage was used as a control. The pellet was washed five times in cold PBS (3 ml per wash) and then resuspended in LB broth (0.5 ml). The solubilized precipitates were added to fresh XL1-Blue cells (0.5 ml of overnight culture) and incubated for 1 hr at 37°C, and aliquots were plated out on LB agar containing chloramphenicol (30  $\mu$ g/ml). Colonies were selected randomly and filter lifts were probed with  $^{125}$ I-labeled BSA-NPN. Additionally, DNA restriction analysis was carried out to determine the presence of heavy- and light-chain inserts.

## RESULTS

**Strategy.** For our purposes it is important to assemble antibody molecules that have correctly folded heavy and light chains and thus retain antigen-recognition capabilities. Previously it had been demonstrated that Fd chains (comprising  $V_H$  and  $C_{H1}$ , the variable region and constant domain 1 of the immunoglobulin heavy chain) and  $\kappa$  chains targeted to the periplasm of *E. coli* assemble to form functional Fab molecules (3, 4). This approach mimics the pathway taken in eukaryotic cells. We reasoned that if the Fd chain was anchored in the membrane and concomitantly provided with secreted  $\kappa$  chain, then functional Fab molecules could form on the membrane surface facing the periplasm. The coexpression behind the *pelB* leader sequence of Fd fused to cpVIII (the major coat protein of M13 phage) and  $\kappa$  chains leads to membrane anchoring of the Fd chain and compartmentalization of the  $\kappa$  chains in the periplasm. These two chains could assemble *in situ* to allow accumulation of functional Fab on the membrane surface, which, by virtue of the cpVIII sequences, would be incorporated along the entire length of the filamentous phage particles on subsequent infection with helper phage. The advantage of the utilization of cpVIII rather than cpIII is twofold: (i) there is potential for multiplicity of binding sites along the particle surface and (ii) this construct should not interfere with phage infectivity (Fig. 1).

**Electron Microscopy.** Examination of filamentous phage and permeabilized cells producing phage revealed specific labeling of phage or exposed bacterial membranes. Phage were observed to contain 1–24 copies of antigen-binding sites per particle. Neither helper phage alone nor intact *E. coli* was labeled with antigen. Background nonspecific binding was very low. Filamentous phage particles emerging from the *E. coli* surfaces were labeled with antigen (Fig. 2).

The generation of a related phage surface expression vector utilizing cpIII as a fusion partner with clone 2b (21) revealed specific antigen labeling of the phage head but not the column. Additionally, human anti-tetanus Fab expressed as a cpIII fusion did not bind to BSA-NPN antigen (C.F.B., unpublished data). The clustering of Fab-cpVIII molecules at the *E. coli* surface supports the "vectorial polymerization" model of phage morphogenesis proposed by Chang *et al.* (22).

**Phage ELISA.** The ELISAs confirmed the presence of functional Fab molecules. Phage-containing supernatant generated from helper phage infection of cells carrying the pCBAK8 construct was titrated in a two-site ELISA using antigen-coated plates and anti-mouse  $\kappa$ -chain enzyme conjugate. For a signal to be generated in this assay the phage particle must (i) have functionally associated Fd and  $\kappa$  chains and (ii) be multivalent. Specificity of the particles was assessed by inhibiting binding to the plate in the presence of free hapten. The phage particles generated exhibited binding to the solid phase of the ELISA and could be inhibited by addition of hapten. Helper phage did not give a signal in the ELISA (Fig. 3).

**Antigen-Specific Precipitation of Phage.** Precipitates were obtained with antibody-containing phage but not helper

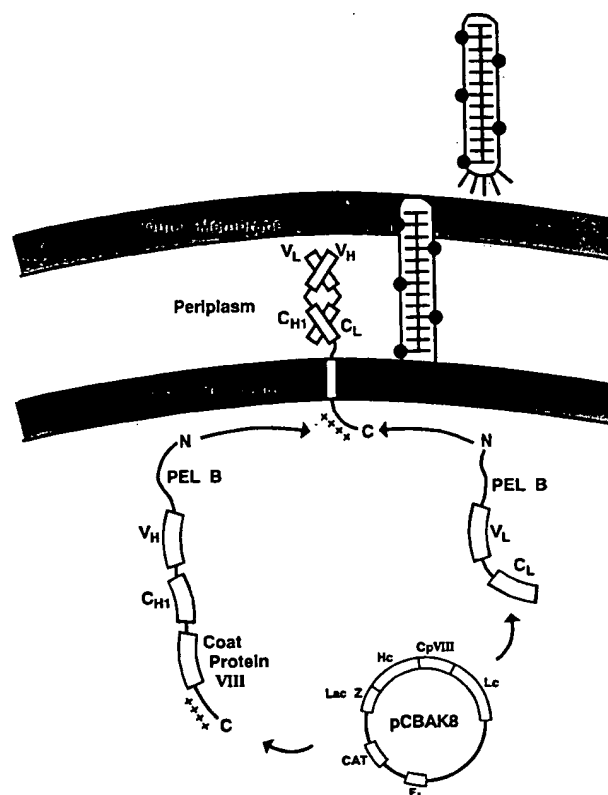


FIG. 1. A cartoon illustrating the composition of CBAK8 vector and the proposed pathway for Fab assembly and incorporation into phage coat. The vector encodes the chloramphenicol acetyltransferase (CAT) marker in addition to the Fd-cpVIII fusion and the  $\kappa$  light chain (Lc). The F1 phage origin of replication facilitates the generation of single-stranded phagemid. Isopropyl  $\beta$ -D-thiogalactoside-induced expression of a dicistronic message encoding the Fd-cpVIII fusion ( $V_H$ - $C_{H1}$ -cpVIII) and the light chain ( $V_L$ - $C_L$ ) leads to the formation of heavy and light chains. Each chain is delivered to the periplasmic space by the PelB targeting sequence, which is subsequently cleaved. The heavy chain is anchored in the membrane by cpVIII fusion, whereas the light chain is secreted into the periplasm. The heavy chain in the presence of light chain assembles to form Fab molecules. The Fab molecules are incorporated into phage particles via cpVIII (●).

phage in the presence of BSA-NPN. In addition, the particles retained infectivity on subsequent incubation with bacterial cells carrying the F' episome and generated  $4 \times 10^5$  colonies from a single solubilized precipitate. All the recovered phage could be shown to bind to antigen and carry the appropriate genetic information. These results give additional evidence for antigen specificity and multivalency. In addition to providing immunological parameters, this precipitation offers possibilities for facile enrichment of antigen-specific phage particles. In principle, phage containing specific antibodies can be highly enriched by precipitation with antigens (which may be cell surface markers or viral, bacterial, or synthetic molecules). The washed antigen-antibody precipitates can be solubilized by the addition of excess antigen and viable phage can be recovered.

For the recovery of rare species an immobilized antigen may be used, which opens the way for differential affinity elution. To demonstrate the utility of immobilized antigen for the enrichment of clones of defined binding specificity, a panning experiment was performed. An ampicillin-resistant phagemid expressing an anti-tetanus Fab as a cpVIII fusion was constructed. Rescue of this clone with helper phage produced phage encoding the ampicillin-resistant phagemid and displaying the anti-tetanus Fab on their coat. These

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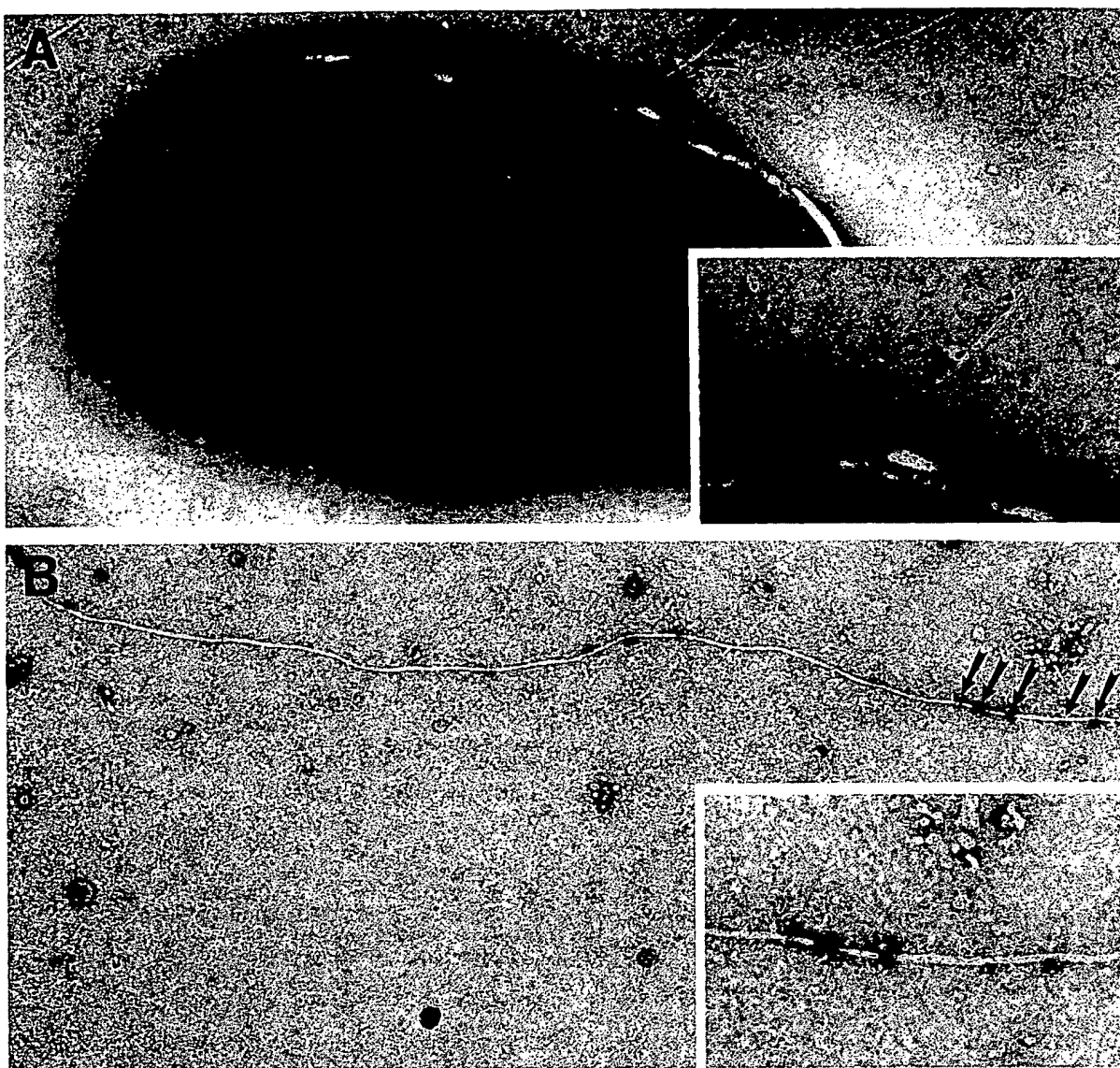


FIG. 2. Electron micrographs showing the localization of 5- to 7-nm colloidal gold particles coated with NPN-BSA conjugate along the surface of filamentous phage. (A) Filamentous phage emerging from the surface of a bacterial cell is specifically labeled with the colloidal gold particles coated with BSA-NPN antigen. ( $\times 54,000$ .) (B) A portion of a mature filamentous phage demonstrating the labeling of antigen-binding sites along its length. ( $\times 54,000$ .)

phage encoding tetanus specificity were mixed 1:100 with phage encoding NPN hapten specificity and allowed to bind to a microtitration plate coated with tetanus toxoid. After a 1-hr incubation, the plate was washed extensively and phage were eluted with a low-pH buffer (15). Infection of logarithmic-phase XL1-Blue cells and plating of aliquots on ampicillin and chloramphenicol plates allowed for direct quantitation of enrichment. Study of over 1000 colonies showed that ampicillin-resistant colonies derived from the eluted phage exceeded chloramphenicol-resistant colonies by 27 to 1. Therefore, panning enriched the phage displaying the anti-tetanus Fab by 2700-fold. This result suggests that a clone of defined specificity present at one part per million will dominate over nonspecific clones following two rounds of panning. The details of this and other enrichment experiments will be the subject of a separate report.

### DISCUSSION

Previously we described a combinatorial vector for the generation of large libraries of antibody Fab fragments in

bacteriophage  $\lambda$ . This approach allowed the production of libraries with  $10^8$ – $10^9$  members. Screening of such libraries by probing filter lifts with  $^{125}\text{I}$ -labeled antigen allowed access to  $10^6$  members (10). We now present a more powerful technique for generating and selecting combinatorial Fab molecules.

In the vector described here, we retained the restriction cloning sites for inserting PCR-generated antibody fragments as previously reported for the  $\lambda$  vector. The rescue of the genes encoding the antibody Fd and  $\kappa$  chains is mediated through the utilization of the fl origin of replication, leading to the synthesis and packaging of the positive strand of the vector on coinfection with helper phage. Since the "mature" virus particle assembles by incorporating the major coat protein around the single-stranded DNA as it passes through the inner membrane into the periplasmic space, not only does it capture the genetic information carried on the phagemid vector but also incorporates several copies of functional Fab along the length of the particle. On subsequent infection of host cells carrying the F' episome the phagemid confers

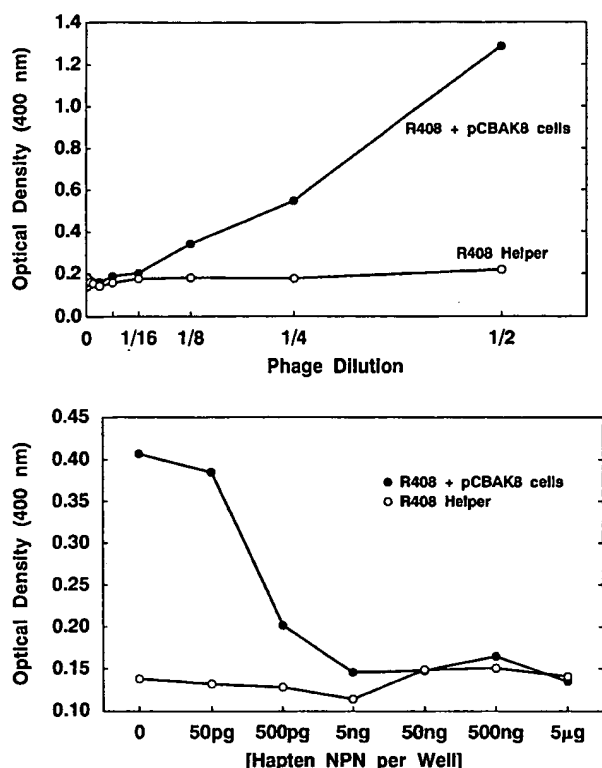


FIG. 3. Characterization of antigen-binding phage. (Upper) ELISA shows serial two fold dilutions of phage containing antibody and helper phage on a BSA-NPN conjugate-coated plate. (Lower) Inhibition ELISA shows that binding of antibody-containing phage to BSA-NPN-coated plates can be inhibited by the addition of free NPN hapten.

resistance, allowing selection of colonies on the appropriate antibiotic. In essence, we have linked the antigen-recognition unit to instructions for its production.

The full power of our earlier combinatorial system could not be fully utilized, since screening allowed ready access to only 0.1–1% of the members. In our phagemid/M13 system we can generate similar-size libraries and access all the members via affinity selection. Further, unlike the  $\lambda$  vector, which generated monovalent Fab, this system generates multivalent particles, thus allowing the capture of a wider range of affinities.

The unique phagemid restriction sites permit the recombination of Fd and  $\kappa$  chains, allowing chain replacement or shuffling. The rescue of filamentous single-stranded DNA allows rapid sequencing and analysis of the genetic makeup of the clone of interest. Indeed, it can be envisaged that phage encoding antibody specificity may be enriched by antigen selection prior to DNA sequencing or mutagenesis. The option to further develop an iterative process of mutation followed by selection may allow the rapid generation of high-affinity antibodies from germ-line sequences. The feasibility of mimicking this "Darwinian" selection utilized by animals can now be tested (23).

Aside from the potential of the system to mimic nature, the phagemid/M13 system would allow a more complete dissec-

tion of the antibody response in humans and thus may yield useful therapeutic and diagnostic reagents.

The membrane anchoring of the heavy chain and the compartmentalization of the  $\kappa$  chain in the periplasm is the key to expressing this functional dimeric protein. The potential of this system is by no means limited to antibodies and may be extended to any protein recognition system or combination of systems containing multiple members. For example, coupling of ligand and effector systems in a high-avidity matrix is now possible. In a similar vein, a library of ligands can be sorted against a library of receptors.

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